REMARKS

Prior to the instant amendment, Claims 61-83 were pending for prosecution in this case. Claims 61, 68, and 72-76 have been amended. Claims 62 and 67 have been canceled without prejudice or disclaimer. Claims 84, 85, and 86 have been added. The amendments to the title, specification, and claims and new Claims 84 to 86 do not constitute new matter and are supported by the specification at, *inter alia*, page 6, lines 10-15; page 32, lines 16-19; and page 35, line 35. Thus, entry of the amendments and new claims is respectfully requested.

Formal matters

Information Disclosure Statement

The copy of the information disclosure statement filed October 10, 2003 and signed by the Examiner on July 18, 2006 is acknowledged. Applicant notes that the Examiner did not initial the foreign patent documents listed on the accompanying Form 1449 and respectfully requests that the Examiner do so to confirm that those documents have been fully considered.

Drawings and sequences

The Examiner objects to certain unspecified drawings and figures under 37 C.F.R. §§ 1.58(a) and 1.83 for consisting only of nucleic acid or protein sequences. Applicant notes, however, that the Figures provide information beyond what is provided by the protein and nucleic acid sequences that have been submitted in computer readable format (i.e., SEQ ID NO:'s) and believes, therefore, that the objection regarding the Figures is improper and should be withdrawn. For example, Figure 1 expressly indicates, via underlined text, the stop and start codons of the disclosed sequence. Such information is not provided in the computer readable sequence listings.

In any event, Applicant notes that the rules cited by the Examiner (37 C.F.R. §§ 1.58(a) and 1.83) were effective as of October 21, 2004 (*see*, 69 FR 56481, Sept. 21, 2004) and, thus, not in effect at the time the instant application was filed. Accordingly, an objection to the Figures based on those rules is not proper in the present situation.

Priority

Please note that due to a previous amendment to the scope of the claims, the Applicant has amended the priority claim for the instant application. No new applications were added to the priority claim. Applicant contends that all applications in the priority claim support the subject matter claimed in the instant application. As can be seen from the amendment on page 2, the instant application claims priority to a series of continuation/continuation-in-part applications through two family lines. The two family lines converge with US application 09/908,827, filed July 18, 2001. US provisional 60/175,481 and PCT/US00/30873 (the '481 and '873 application, respectively) are the earliest application filed in each family line. The '481 and '873 applications each contain disclosures that supports the currently claimed subject matter as follows.

60/175,481, filed January 11, 2000

Applicant believes that the '481 application provides support for the instantly claimed subject matter through, at least, the following disclosures:

- SEQ ID NO:2
- page 68, lines 15-19 regarding the use of anti-PRO10272 (IL-17E) antibodies in the treatment of various inflammatory diseases, certain autoimmune disorders, and various bone disorders involving bone resorption; and
- page 24, line 29 through page 25, line 7 regarding antagonist antibodies and their fragments contemplated as suitable antagonist molecules;

PCT/US00/23328, filed August 24, 2000 ("the '328 application")

Applicant believes that that '873 application provides support for the instantly claimed subject matter through, at least, the following disclosures:

- SEQ ID NO:156
- page 24, lines 8-16 regarding antagonist antibodies and their fragments contemplated as suitable antagonist molecules.
- page 97, line 27 regarding the teaching that PRO10272 (IL-17E) binds to PRO5801 (RH1).

Because the '481 and '328 applications are the earliest applications filed in each of the two family lines, each of the subsequently filed applications in those lines contain at least as much disclosure (*i.e.*, through incorporation by reference) regarding the currently claimed subject matter. Accordingly, Applicant does not believe it is necessary to provide page and line citations from all of the subsequently-filed priority documents but will do so at the Examiner's request.

Rejections under 35 U.S.C. § 112, second paragraph – indefiniteness

Claims 61, 64-74, and 76-83 stand rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter. Claim 61 is considered indefinite for failing to specifically identify the structure of a PRO10272 polypeptide. Claim 76 is considered indefinite for failing to define the metes and bounds for the recitation "at least about 85%." The remaining claims are considered indefinite and, therefore, stand rejected for depending from Claims 61 and 76.

Applicant believes that in light of the amendment to Claim 61 to recite the "polypeptide of SEQ ID NO:6" and the amendment to Claim 76 to recite "at least 85%" the indefiniteness rejections are moot and should be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph – new matter

Claims 73 and 74 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention at the time the application was filed. Specifically, the Examiner could not locate in the specification the basis for the claims to a pharmaceutical composition comprising an immunosuppressant, wherein the immunosuppressant is methotrexate.

The subject matter of Claims 73 and 74 do not constitute new matter and are supported in the specification at, *inter alia*, page 6, lines 10-15 and page 35, line 35. To expedite prosecution, the Applicant has amended claim 73 to recite an "immune inhibiting molecule" as opposed to an "immunosuppressant." Immune inhibiting molecule a broad term that encompasses the term immunosuppressant and is disclosed in the specification at, *inter alia*, page 6, line 10. In

addition, methotrexate is disclosed at page 35, line 35. It was well-known in the art at the time of filing that methotrexate is an immune inhibiting molecule. (*See, e.g.*, Genestier, L., *et al.*, J. CLIN. INVEST. 102(2): 322-28 (1998) (e.g., Introduction, last paragraph), a copy of which has been provided with this response). Accordingly, in light of the teaching of the specification and knowledge in the art at the time of invention, the Applicant believes that the new matter rejection of Claims 73 and 74 is improper and should be withdrawn.

Rejections under 35 U.S.C. § 112, first paragraph – enablement

Claims 61, 75, and the dependent Claims 62-74 stand rejected under 35 U.S.C. § 112, first paragraph, as failing to reasonably provide enablement for claims to a method of treatment using an antagonist of a PRO10272 polypeptide or a kit comprising a composition comprising an antagonist of the PRO10272 polypeptide of SEQ ID NO:6. Specifically, the Examiner argues that the claim to "an antagonist" reads on any or all polypeptides possibly associated with a degenerative cartilaginous disorder and encompasses a genus of molecules with broad structural diversity not sufficiently disclosed in the specification of the instant application. Although the Applicant believes that the specification enables one of ordinary skill in the art to practice a method of treatment using antagonists to the PRO10272 polypeptide of SEQ ID NO:6 in addition to antibodies (*see, e.g.*, page 31, lines 23-31), in the interest of expediting the examination of the instant application, Applicant amends Claims 61 and 75 to be directed to antagonist antibodies or fragments thereof. Accordingly, in light of the amendments to Claims 61 and 75, the Applicant believes that the rejection of Claims 61 and 75 is moot and respectfully requests that the rejection be withdrawn. Indeed, the Examiner has acknowledged that the specification enables claims directed to a method of treatment using an antagonist antibody to the PRO10272 polypeptide of SEQ ID NO:6. (*See,* Office Action, page 5, ¶2).

Rejections under 35 U.S.C. § 112, first paragraph – written description

Claims 61, 75, and the dependent Claims 62-74 stand further rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed invention at the time the application was filed. Specifically, the

Examiner appears to be asserting that the recitation of "PRO10272 polypeptide" and generic "antagonist" reads on a functional equivalent of any or all molecules having an antagonizing activity for which there is no § 112 support in the specification. While the Applicant does not agree with the Examiner's position, in line with the response to the § 112, enablement rejection, the Applicant has amended Claims 61 and 75 in the interest of expediting prosecution. For example, the amended Claims 61 and 75 are directed to antagonist antibodies or fragments thereof of the polypeptide of SEQ ID NO:6. Applicant believes that such amendments render the written description rejection moot and requests that the rejection be withdrawn.

Rejection Under 35 U.S.C. § 102(e)

Claims 72 and 75 are rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent No. 6,562,578 by Gorman *et al.* (hereinafter referred to as "Gorman *et al.*"). In support of the rejection, the Examiner notes that Gorman *et al.* discloses a human IL-17 like cytokine, designated as IL-174 and encoded by SEQ ID NO:14 (161 amino acids in length), which comprises the amino acids 19-177 of the present SEQ ID NO:6 (177 amino acids in length) with 100% sequence identity. The Examiner also asserts that Gorman *et al.* teaches antibodies to IL-174, including chimeric or humanized antibodies, and, *inter alia,* compositions and kits for therapeutic use. Applicant traverses the rejection for the following reasons.

Applicant submits that Gorman *et al.* cannot anticipate the present claims under the standards that govern § 102(e). The proper standard for anticipation under § 102(e) is that articulated by the Court of Customs and Patent Appeals in *In re Wertheim and Mishkin,* 646 F.2d 527, 209 USPQ 554 (CCPA 1981): namely, that a reference patent or published patent application can anticipate under 35 U.S.C. § 102(e) as of a particular date only to the extent that there is a sufficient disclosure under 35 U.S.C. § 112, first paragraph, for the subject matter at issue. *See also* MPEP 2136.03, subheading IV. As explained below, Applicant submits that Gorman *et al.* does not support the present claimed subject matter under § 112 and, therefore, cannot be a proper § 102(e) reference. Applicant notes that the legal standard for § 102(b) articulated by statute and the case law (i.e., *In re Schoenwald*) cannot be used to support a rejection under § 102(e).

Specifically, in *Wertheim*, the Court held that in order for the patent to enjoy prior art status under § 102(e), the application to which priority is claimed must satisfy the disclosure requirements of the first paragraph of § 112 for the claimed subject matter at issue. *See Wertheim*, 646 F.2d at 537. It is entirely consistent with the logic of this provision that the *actual* patent disclosure (*i.e.*, the disclosure that is in the application that led to the patent, rather than an earlier application to which a claim under § 120 is made) must meet the requirements of § 112, first paragraph, for the subject matter being rejected. Accordingly, if the Gorman *et al.* patent fails to satisfy the requirements of § 112, including that the disclosure teach how to use a claimed invention, it cannot be a proper § 102(e) reference.

Applicant contends that Gorman *et al.* fails to disclose any information that describes in any credible manner any specific biological role, function or activity associated with the IL-174 polypeptide. Instead, the disclosure speculates as to possible functions or activities that the polypeptide might possess. Even in this respect, the Gorman *et al.* disclosure makes these representations in a way that is so generalized and abstract as to be meaningless to a person of ordinary skill in the art. For example, Gorman *et al.* merely proposes to one of ordinary skill in the relevant art that IL-174 is but one of several members of peptides in the IL-17 family of cytokines related to TGF-β. (*See* Gorman *et al.*, Col. 6, ll. 23-29). Yet, there is no further, specific disclosure regarding what role, if any, IL-174 plays as a member of the IL-17 family of cytokines or, for example, to what receptor (or any other biological substrate) it interacts with. This absence of experimental data for the IL-174 polypeptide renders the disclosure of Gorman *et al.* incapable of establishing, under § 101, a specific, substantial, and credible utility for the polypeptide. In turn, as Gorman *et al.* fails to satisfy § 101 for the underlying polypeptide, its disclosure does not satisfy § 101 utility for the pharmaceutical compositions and kits comprising antagonist antibodies to the polypeptide of claims 72 and 75.

Based on substantial precedent (*See, e.g., In re Zeigler, In re Brana, and In re Fouche)*, if Gorman *et al.* fails to satisfy § 101 for the presently claimed subject matter, it fails to meet the requirements of § 112, first paragraph. In particular, the insufficient disclosure in Gorman *et al.* regarding the IL-174 polypeptide (i.e., the disclosure does not set forth a specific, substantial and credible utility methods of using antagonist antibodies and compositions thereof), makes it impossible for Gorman *et al.* to satisfy the "how to use" prong of § 112 for the presently claimed

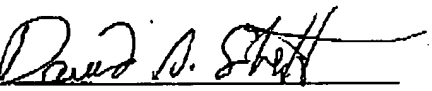
subject matter. As a matter of law, because Gorman *et al.* fails to satisfy the requirements of § 101, it fails to provide an enabling disclosure under § 112 and cannot anticipate the claims under § 102(e). Applicant asks that the rejection of the claims under § 102(e) in view of Gorman *et al.* be withdrawn.

In light of the above amendments and remarks, Applicant believes that this application is now in condition for immediate allowance and respectfully requests that this case pass to issue.

The Examiner is invited to contact the undersigned at (202) 736-8157 if any issues may be resolved in that manner.

Respectfully submitted,

Date: January 16, 2007

By: 

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Immunosuppressive Properties of Methotrexate: Apoptosis and Clonal Deletion of Activated Peripheral T Cells

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Abstract

The folate antagonist methotrexate (MTX) is extensively used in graft-versus-host disease, rheumatoid arthritis, and other chronic inflammatory disorders. In addition to its antiinflammatory activity associated with increased release of adenosine, MTX exerts antiproliferative properties by inhibition of dihydrofolate reductase and other folate-dependent enzymes. However, the mechanisms of immunosuppressive properties associated with low-dose MTX treatments are still elusive. We report here that MTX (0.1–10 μM) induces apoptosis of in vitro activated T cells from human peripheral blood. PBL exposed to MTX for 8 h, then activated in drug-free medium, underwent apoptosis, which was completely abrogated by addition of folinic acid or thymidine. Apoptosis of activated T cells did not require interaction between CD95 (Fas, APO-1) and its ligand, and adenosine release accounted for only a small part of this MTX activity. Apoptosis required progression of activated T cells to the S phase of the cell cycle, as it was prevented by drugs or antibodies that interfere with IL-2 synthesis or signaling pathways. MTX achieved clonal deletion of activated T cells in mixed lymphocyte reactions. Finally, in vitro activation of PBL taken from rheumatoid arthritis patients after MTX injection resulted in apoptosis. Altogether, the data demonstrate that MTX can selectively delete activated peripheral blood T cells by a CD95-independent pathway. This property could be used as a new pharmacological end point to optimize dosage and timing of MTX administration. It may account for the immunosuppressive effects of low-dose MTX treatments. (*J. Clin. Invest.* 1998. 102:322–328.) Key words: methotrexate • apoptosis • T lymphocytes • thymidylate synthase • rheumatoid arthritis

Introduction

Methotrexate (MTX)[1] is a folate antagonist first developed for the treatment of malignancies (1) and, subsequently, used in nonneoplastic diseases as an antiinflammatory and/or immunosuppressive drug. MTX is currently the most commonly used treatment of rheumatoid arthritis (2, 3), and other chronic inflammatory disorders. MTX is also effective in the prophylaxis of acute graft-versus-host disease either alone or in association with cyclosporin A (CsA) and/or prednisone (4–6) or FK506 (7). MTX has also been used as an adjunct therapy for persistent mild cardiac allograft rejection (8). Most pharmacological studies have addressed the use of MTX in cancer chemotherapy, where doses could be escalated up to 30 g/m² by administration of the antidote leucovorin (folinic acid, citrovorum factor). In autoimmune diseases and allografts, however, MTX dosage is usually in a range of 7–15 mg per week, given orally or by intramuscular injections.

Biochemical pharmacology studies of MTX in tumor cell lines by J. Jolivet, B.A. Chabner, and co-workers have shown that MTX, like physiological folates, is converted to polyglutamate forms that are not readily transported across the cell membrane (9–11). Those polyglutamated derivatives not only inhibit dihydrofolate reductase (DHFR), the major MTX target, but also have markedly increased affinity for certain folate-dependent enzymes such as thymidylate synthase (TS), 5-amino imidazol-4-carboxamide ribonucleotide transformylase, and the triple complex of enzymes that interconvert various forms of reduced folate (11, 12). The enzyme responsible for MTX conversion, folylpolyglutamyl synthetase, catalyzes the addition of γ-linked glutamate groups to the end carboxyl group of the neighboring folyl glutamate, using ATP as its energy source. The activity of this enzyme, first demonstrated in erythrocytes and then in human liver, was shown to vary among tumor cell lines. Surprisingly, the polyglutamation of MTX in normal tissues, including the lymphoid system, has received little attention so far, and the sensitivity of resting or activated peripheral T cells to growth inhibition and, eventually, apoptosis in the presence of MTX concentrations, achieved during low-dose treatment, has not been investigated.

The present study addressed the in vitro activity of MTX on human PBL. We report here that MTX selectively induces apoptosis of activated but not resting lymphocytes, even after short term exposure to MTX and subsequent activation in drug-free medium, thus, providing the first evidence for an immunosuppressive activity of low-dose intermittent MTX administration. Selective susceptibility of activated T cells in the S/G2 phase of the cell cycle may result in clonal deletion of T cells that are activated by antigen at the time of MTX administration.

L. Genestier and R. Paillot contributed equally to this work.
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1. *Abbreviations used in this paper:* ADA, adenosine deaminase; CsA, cyclosporin A; DHFR, dihydrofolate reductase; MLR, mixed lymphocyte reactions; MTX, methotrexate; RPM, rapamycin; TS, thymidylate synthase; TUNEL, Tdt-mediated dUTP-FITC nick end labeling.

Methods

Reagents and monoclonal antibodies. MTX, folic acid, folinic acid, adenosine, thymidine, adenosine deaminase (ADA), α,β-methylene adenosine-5'-diphosphate (ACPD), PHA, PMA, ionomycin, concanavalin A, and *Staphylococcus aureus* enterotoxin B were obtained from Sigma Chemical Co. (St. Louis, MO). Rapamycin (RPM) and FK506 were a gift from A. Altman (La Jolla Institute for Allergy and Immunology, La Jolla, CA), and CsA was supplied by Sandoz Pharmaceutical Division (Novartis, Paris, France). The CD3 mAb OKT3 was from Cilag Laboratories (Levallois-Perret, France). The CD25 mAb ARIL-2 (IgG1) and anti-thymocyte globulins were a gift from 7 Alberici (Pasteur-Merieux, Lyon, France). The agonist (CH11, IgM) anti–human CD95 mAb was from Coulter-Immunotech (Marseille, France). The antagonist anti–human CD95 mAb ZB4 (IgG1) was from Kamiya Biomedical (Thousand Oaks, CA). The CD25, CD69, and CD95 mAbs (fluorescein isothiocyanate conjugates) were from Becton Dickinson (Mountain View, CA) and Immunotech (Marseille, France), respectively.

Cell preparation and culture. PBL were collected from healthy donors in the presence of sodium citrate. Blood was defibrinated, and then mononuclear cells were isolated by centrifugation on a layer of Histopaque® (Sigma Chemical Co.). Those cell suspensions, referred to as PBL, contained 1.8±0.4% monocytes, as defined by CD14 expression. PBL were resuspended in Rosewell Park Memorial Institute culture medium (RPMI 1640; Sigma Chemical Co.), supplemented with 10% FCS or TCH medium (serum free; ICN, Orsay, France), 2 mM L-glutamine, and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml). Cultures were maintained at 37°C in a humid atmosphere containing 5% CO_2. During the last 8 h of incubation they were pulsed with (methyl-^{3}H)thymidine ([^{3}H]TdR; Amersham Int., Little Chalfont, UK) at 0.5 μCi/well. ^{3}H-TdR uptake was measured using a Packard direct beta counter (Meriden, CT) after harvesting. For mixed lymphocyte reactions (MLR), the human B lymphoma cell lines RAJI and DAKIKI were used as stimulators (13). Stimulator cells were treated for 1 h at 37°C with 25 μg/ml of mitomycin C (29805; Serva Heidelberg, Germany), extensively washed, and then mixed with PBL at a ratio of 1 B cell for 10 PBL.

Measurement of nuclear apoptosis. After 3 d of culture, PHA-activated PBL were harvested. Dead cells were removed by centrifugation on a layer of Histopaque® (Sigma Chemical Co.). Viable cells (10^6/ml) were washed in HBSS, and then incubated in 96-well microplates (Costar, Cambridge, MA) with MTX. In other experiments, PBL were either incubated for 1–24 h in the presence of MTX, and then activated with PHA for 24 to 72 h, or MTX and PHA were added together at the onset of the culture. Cell death was evaluated by fluorescence microscopy after staining with Hoechst 33342 (Sigma Chemical Co.) at 10 μg/ml after previously described methods (14). Apoptosis was also measured by flow cytometry after addition of biotinylated annexin V (Boehringer Mannheim, Indianapolis, IN; 15) and by TdT-mediated dUTP–FITC nick end labeling (TUNEL), as previously described (16), using reagents from Boehringer Mannheim. Samples were analyzed by flow cytometry on a FACScan® (Becton Dickinson). Nuclear fragmentation and/or marked condensation of the chromatin with reduction of nuclear size were considered as typical features of apoptotic cells. Based on these measurements, results were expressed as percentage of apoptotic cells or percentage of specific apoptosis according to the following formula: specific apoptosis = (T − C)/(100 − C), where T stands for % of apoptotic-treated cells and C for % of apoptotic control cells.

The morphological features of the cells after MTX treatment were also observed by transmission electronic microscopy, as previously described (14). For DNA fragmentation assay, cells were incubated in RPMI medium for 12 h with MTX, and DNA preparations were obtained and processed for electrophoresis in 2% agarose gel after previously described methods (17).

Immunofluorescence assays. After 1 d of culture, cells were washed with isotonic NaCl/Pi buffer containing 1% BSA and 0.2% NaN_3 (PBS/BSA/azide), and then incubated with 10 μl of fluorescein isothiocyanate conjugated CD25, CD69, or CD95 mAb per 5×10^5 cells for 30 min at 4°C. After two washes in PBS/BSA/azide, cell were fixed with 1% formaldehyde in PBS/BSA/azide. Lymphocytes were identified by cell size (small angle light diffraction) and this fraction was gated for analysis, thereby excluding dead cells but including small lymphocytes and blasts. To evaluate the mitochondrial transmembrane potential ($\Delta\psi_m$), cells (5×10^5/ml) were incubated with 3,3'-dihexyloxacarbocyanine ($DiOC_6(3)$) 40 nM in PBS (Molecular Probes, Inc., Eugene, OR) for 15 min at 37°C, followed by flow cytometry analysis (λ. Ex. Max., 488 nM; λ. Em. Max., 525 nm). The decrease in $\Delta\psi_m$ is a characteristic of apoptotic cells (18).

CD95-ligand mRNA quantification was carried out following methods described in detail in another report (19).

Patients. Peripheral blood from one patient with a defect in the CD95 apoptotic signaling pathway was kindly made available by Alain Fischer (Hôpital des Enfants Malades, Paris, France). Details of the phenotypic alterations of this patient have been previously reported (20). Six patients with rheumatoid arthritis treated by weekly intramuscular injections of MTX (7.5 mg in one patient, 12.5–15 mg in the five others) in the clinical immunology unit, Hôpital E. Herriot (patients of P. Miossec) gave their informed consent for blood sampling (5 ml) before, and 6, 24, and 48 h after MTX injection. Cells were cultured with or without PHA.

Results

MTX induces apoptosis of mitogen-activated but not resting peripheral T cells. PBL were activated by PHA for 3 d, and then dead cells were removed by centrifugation, and viable cells were incubated for 15 h with MTX (1 μM). Apoptosis was demonstrated by internucleosomal fragmentation resulting in a typical "ladder" of 180-bp fragments and multiples thereof in agarose gel electrophoresis, whereas such fragments were not detected in nonactivated PBL (Fig. 1 *A*). Apoptosis of PHA-activated cells, but not resting PBL was confirmed by typical condensation or fragmentation of cell nuclei, as revealed by Hoescht 33342 staining in fluorescence microscopy (Fig. 1 *B*), electron microscopy (Fig. 1 *C*), by the decrease of mitochondrial transmembrane potential ($\Delta\psi_m$) (Fig. 1 *D*), and the presence of DNA breaks revealed by the TUNEL assay, (Fig. 1 *D*).

The kinetics of apoptosis showed a progressive increase up to 16 h (10 μM) or 28 h (0.1 μM) (data not shown). Similar observations were made with PBL activated by PMA plus ionomycin, concanavalin A, the CD3 mAb OKT3, polyclonal rabbit anti-thymocyte globulins and *S. aureus* enterotoxin B, but not by PMA alone (data not shown). We concluded that MTX (1 μM) induced apoptosis of activated T cells. A dose range of MTX concentrations was tested, demonstrating that apoptosis occurred at 0.1–10 μM, but not at 100 μM (Fig. 2 *A*). Counts of viable cells for 4 d after removal of PHA showed a progressive increase in controls (without MTX), a borderline decrease in the presence of MTX at 0.01 and 100 μM, and a considerable loss of viable cells with MTX at either 1 or 10 μM (Fig. 2 *B*). When compared with measurements of apoptosis, these data indicate that MTX at low (0.01 μM) and high (100 μM) concentrations inhibits cell proliferation without inducing apoptosis, whereas at intermediate concentrations (from 0.1 to 10 μM), the decrease of viable cells is mostly accounted for by apoptosis. Addition of MTX at the onset of PHA activation resulted in a marked progressive decrease of viable cell counts in the presence of MTX (from 0.1 to 10 μM), whereas at low (0.01 μM) and high (100 μM) concentrations, MTX inhibited

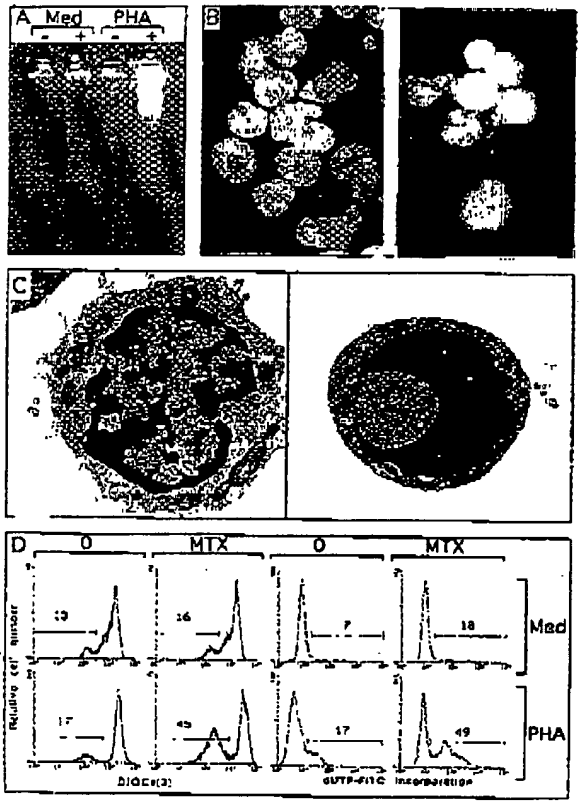

Figure 1. MTX induces apoptosis of activated but not resting T cells. PBL were incubated 3 d with medium or PHA (5 μg/ml). Dead cells were removed and viable cells were treated for 15 h with MTX (10^{-6} M). (*A*) DNA fragmentation was evaluated by electrophoresis on 2% agarose gel (Medium and PHA, without (−) or with (+) MTX). (*B* and *C*) Morphology of activated PBL after Hoescht 33342 staining and by electron microscopy, respectively (without MTX (*left*) or with MTX (*right*)). (*D*) Alteration of the mitochondrial transmembrane potential ($\Delta\psi_m$) measured by staining with $DiOC6_{[3]}$ and DNA strand breaks detection by the TUNEL assay as described in Methods. Numbers in each histogram refer to the percentage of cells below ($DiOC6_{[3]}$) or above (TUNEL) the threshold of normal controls.

cell proliferation but induced only a moderate and/or delayed loss of viable cells (data not shown).

PBL incubated with MTX undergo apoptosis upon subsequent activation in drug-free medium. Preincubation of PBL with MTX (from 0.1 to 50 μM) resulted in apoptosis on subsequent PHA activation for 24 h (Fig. 3 *A*), and maximal apoptosis was attained after 8 h incubation with MTX (data not shown). To determine the half-life of MTX apoptosis–inducing activity, PBL were incubated with MTX (1 μM) and, subsequently, cultured in drug-free medium for various time intervals before activation by PHA. Results indicate a progressive linear decrease of the percentage of apoptotic cells with an average half-life of 3 d (Fig. 3 *B*). Counts of viable cells after PHA activation in drug-free medium demonstrated that MTX inhibits PHA-induced cell proliferation at 0.01 and 100 μM,

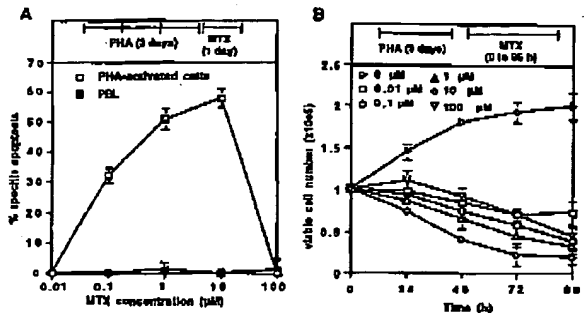

Figure 2. Characteristics of MTX-induced apoptosis. (*A*) Dose response: resting T cells (■) or cells activated by PHA (5 μg/ml) during 3 d (□) were treated with a dose range of MTX. Apoptotic cells were evaluated after 24 h. Values are the mean±SEM of three independent experiments. (*B*) Effect of MTX on viable cell recovery. PBL were stimulated with PHA (5 μg/ml) without or with MTX (10^{-8} M to 10^{-4} M) added at day 3. Viable cell number was determined by trypan blue exclusion at the indicated times. Values are the mean±SEM of three independent experiments.

and induced a transient decrease of viable cell numbers at intermediate (0.1–10 μM) concentrations (data not shown).

MTX-induced apoptosis of activated T cells results from DHFR and TS inhibition. Knowing that MTX and its polyglutamated derivatives can interfere with several folate-dependent enzymes, we determined whether blockade of the two major targets of MTX activity, DHFR and TS, could account for MTX-induced apoptosis of activated T cells. To this end, 3-d PHA–activated cells were incubated with MTX (1 μM) together with folic acid, folinic acid, or thymidine. Folinic acid showed dose-dependent inhibition of MTX activity, with ~ 80% decrease at 1 μM (Fig. 4 *A*). Folic acid was ~ 10–50 times less effective on a molar basis, and complete inhibition required 100 μM. Increasing the extracellular concentration of

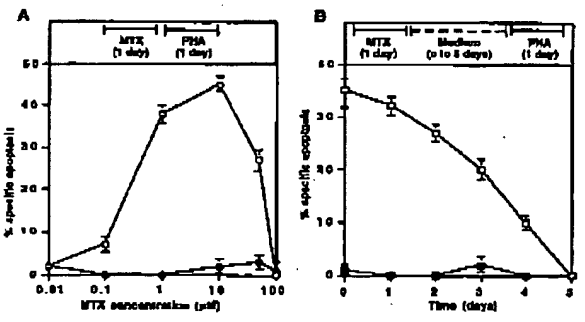

Figure 3. Apoptosis induced by MTX pretreatment: dose response (*A*) and half-life of intracellular MTX (*B*). PBL were incubated with a dose range of MTX (*A*) or MTX (10^{-6} M) for 24 h (*B*). After removing the drug by two washes in HBSS, cells were immediately activated by PHA (5 μg/ml) (*A*) or maintained between 1 and 5 d in medium before PHA activation (*B*). Percent apoptotic cells were evaluated at 24 h in control cell suspensions incubated in medium alone (*closed symbols*) or in cells cultured with PHA (5 μg/ml) (*open symbols*). Results are expressed as percent-specific MTX apoptosis. Values are the mean±SEM of four individual experiments.

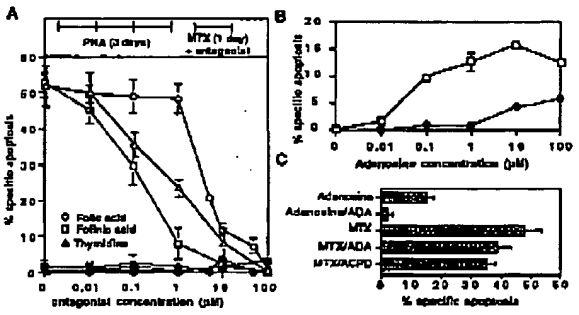

Figure 4. Effect of folic acid, folinic acid, or thymidine (*A*); adenosine deaminase and ACPD on apoptosis induced by adenosine or MTX (*C*). Resting T cells (*closed symbols*) or cells activated by PHA (5 μg/ml) in serum-free medium for 3 d (*open symbols*) were treated with MTX (10^{-6} M) plus a dose range of folic acid, folinic acid, or thymidine (*A*) or with adenosine in serum-free medium. Identical values were observed in the presence of ACPD (10 μM) (*B*). 3-d PHA-activated cells were incubated with ADA (2 U), or ACPD (10 μM), and treated 24 h with MTX (10^{-6} M) or adenosine (10^{-6} M) (*C*). Apoptotic cells were evaluated after 24 h, and results are expressed as percent-specific apoptosis as described in Methods. Values are the mean ± SEM of three independent experiments.

thymidine to 10 μM was sufficient to prevent 90% of MTX-induced apoptosis (Fig. 4 *A*).

Adenosine plays a limited role in MTX-induced apoptosis. Knowing that adenosine represents the major mediator of the antiinflammatory activities of MTX (21), we investigated its contribution to MTX-induced apoptosis. Addition of adenosine (0.1–1 μM) in serum-free medium with or without ACPD resulted in apoptosis of ~ 15% of PHA-activated cells (Fig. 4 *B*). At higher concentrations (10–100 μM), adenosine induced some apoptosis of unactivated lymphocytes, but the percentage of apoptotic PHA-activated cells did not increase. The apoptotic activity of adenosine (1 μM) was completely abrogated by adenosine deaminase, whereas that of MTX was only marginally decreased (Fig. 4 *C*). It was concluded that sensitivity to adenosine-mediated apoptosis was restricted to a subset of PHA-activated cells, accounting for ~ 15–20% of cells susceptible to MTX.

MTX-induced apoptosis requires progression to the S phase of the cell cycle. The apoptotic signal triggered by MTX is likely to be initiated at the time of DNA synthesis, during the S phase of the cell cycle. Supporting this hypothesis, the percentage of blasts and the cell surface expression of CD69, CD25 (α chain of the IL-2 receptor), and CD95 (Fas, APO-1), that are typical markers of activated T cells in the G1 phase of the cell cycle were not decreased by MTX (Fig. 5 *A*). Furthermore, inhibition of IL-2 synthesis by CsA or FK506, interference with IL-2 receptor signaling by rapamycin, and competition with IL-2 binding to its receptor by a CD25 mAb, all markedly decreased [^{3}H]TdR incorporation and reduced MTX-induced apoptosis in parallel (Fig. 5 *B*). None of the inhibitors used to interfere with the IL-2 pathway increased the basal level of apoptosis in the absence of MTX.

MTX-induced apoptosis does not involve CD95L/CD95 interaction. Activated T and B lymphocytes express the death-signaling receptor CD95 (Fas, APO-1), which mediates apoptosis upon oligomerization. Because MTX was reported to stimulate CD95 ligand (CD95L) mRNA expression in human leukemia T cell lines (22), we studied the possible contribution of CD95L/CD95 interaction in the apoptotic effect of MTX. Expression of CD95L mRNA by 3-d PHA–activated cells could be readily induced by the association of a phorbol ester with a calcium ionophore, whereas MTX (10 and 200 μM) triggered only borderline CD95L gene transcription (Fig. 6 *A*). Apoptosis induced by MTX was not altered by addition of the CD95 antagonist antibody ZB4, which completely inhibits apoptosis induced by CD95L or by the CD95 agonist antibody CH11 (Fig. 6 *B*). PBL rapidly express CD95 upon activation, but their sensitivity to CD95-dependent apoptosis progressively develops from day 3 to day 6 of mitogenic activation, and requires IL-2 (23). After 24 h of mitogenic activation, PBL were fully susceptible to MTX-induced apoptosis (Figs. 3 *B* and 6 *C*), yet resistant to the CD95 agonist antibody CH11. Finally, PBL from a patient with normal CD95 expression but defective CD95 signaling pathway, as shown by their resistance to CH11-induced apoptosis, were fully susceptible to MTX (Fig. 6 *D*). It was concluded that MTX-induced apoptosis of mitogen-activated cells occurred through a CD95-independent pathway.

MTX induces clonal deletion of alloreactive T cells. Alloreactive T cells undergo activation and clonal expansion when cocultured with cells that express different MHC class II molecules, a reaction termed MLR (mixed lymphocyte reaction) (24). Subsequent culture with the same stimulator cells results in accelerated proliferation, as shown by a peak of [^{3}H]TdR incorporation and CD25 expression at 2–3 d, whereas the response to third party stimulator cells follows primary kinetics with maximal cell proliferation occurring after 5–6 d (13). Such differences indicate that the two types of stimulator cells are

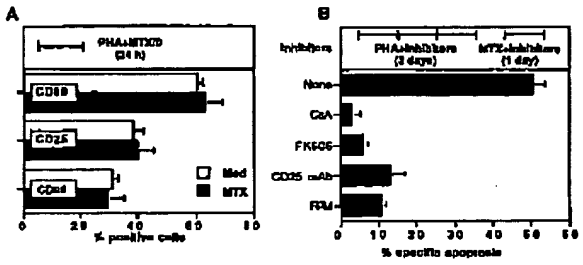

Figure 5. MTX-induced apoptosis and progression of activated PBL in the S phase of the cell cycle. PBL were activated by PHA (5 μg/ml) with or without MTX. (*A*) Percentage of cells expressing G1 phase markers (*CD69, CD25, CD95*) among PHA-activated PBL after 24 h in the presence (*gray bands*) or absence (*white bands*) of MTX (10^{-6} M). (*B*) Effect of inhibition of the G_1 to S phase transition on MTX-induced apoptosis. PBL were activated by PHA (5 μg/ml) with or without CsA (250 ng/ml), FK506 (10 nM), CD25 mAb (10 μg/ml), and RPM (60 nM). Results are expressed as the specific MTX apoptosis, as described in Methods. Values are the mean ± SEM of four independent experiments. [^{3}H]TdR incorporation was determined during the last 8 h of culture with PHA: mean dpm (stimulation index in brackets). Nonactivated PBL: 300, PHA alone: 5,250 (17.5), PHA + CsA: 1,280 (4.2), PHA + FK506: 1,420 (4.7), PHA + CD25 mAb: 2,050 (6.8), PHA + RPM: 1,300 (4.3).

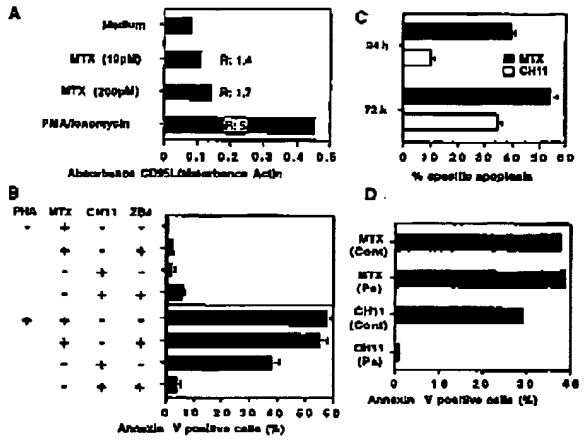

Figure 6. MTX-induced apoptosis does not involve CD95/CD95L interaction. PBL were cultivated for 3 d in the presence of PHA (5 μg/ml). Dead cells were eliminated by centrifugation on Ficoll, and viable cells were incubated with MTX (10 or 200 μM) or with PMA (10 ng/ml) plus ionomycin (0.5 μg/ml) for 12 h. mRNA of each sample was amplified by reverse transcriptase–PCR as described in Methods, and separated on 2% agarose gel. Results are expressed as the ratio: absorbance of CD95L/absorbance of actin. *R* corresponds to the ratio: absorbance assay/absorbance of medium. (*B*) Effect of the antagonist Fas mAb ZB4 on MTX-induced apoptosis. 3-d PHA–activated cells were incubated 1 h with ZB4, and then treated with medium, MTX (10^{-5} M), or the agonist CD95 mAb CH11 (1 μg/ml) for 24 h, and percentage of apoptotic cells was determined by annexin V binding. (*C*) Sensitivity to apoptosis induced by MTX and CD95 mAb CH11 according to culture duration. 1-d or 3-d PHA–activated cells were treated by MTX (10^{-6} M) or CD95 mAb CH11 (1 μg/ml) for 24 h. The percentage of apoptotic cells was determined by flow cytometry after staining with annexin V. Results are expressed as mean±SEM of four independent experiments. (*D*) MTX-induced apoptosis in lymphocytes from a patient with a CD95-signaling defect. 3-d PHA–activated cells were treated with MTX (10^{-6} M) or CH11 (1 μg/ml). After 12 h of incubation, the percentage of apoptotic cells was determined by flow cytometry after staining with annexin V.

recognized by distinct T cell clones, although cross-reactivity and/or HLA allele sharing between stimulator cells may often result in intermediate kinetics. To investigate whether a brief exposure to MTX could selectively delete activated T cells without impairing the functional capacities of other T cells in the same culture, we added MTX (1 μM) during the last 16 h of a primary MLR. Cells were then incubated in fresh medium for 4 d to allow the decrease of intracellular MTX in accord with the kinetics shown in Fig. 3 *C*, and the viable cells were then cocultured with either the same or a different allogeneic B cell line. In these conditions, the secondary response to identical stimulator cells was nearly completely abrogated, whereas the response to a third party cell line was not affected (Fig. 7), indicating that the effect of MTX was clonally restricted to the T cells that were activated at the time of exposure to the drug.

Lymphocytes from MTX-treated patients undergo apoptosis upon in vitro activation. We verified in six rheumatoid arthritis patients, that a single injection of MTX during low-dose treatment was sufficient to prime lymphocytes to apoptosis upon subsequent mitogenic activation ex vivo (Fig. 8). In vitro apoptosis, determined after 24 h of activation by PHA, was completely inhibited in the presence of folinic acid (10 μM) and folic acid (100 μM) (data not shown), indicating that it could be attributed to MTX or its polyglutamated derivatives.

Discussion

MTX was shown to exert a wide range of antiinflammatory activities (21, 25–27) that are primarily mediated by the release of adenosine from different cell types that express ecto-5′-nucleotidase (28). Such antiinflammatory properties do not exclude a genuine immunosuppressive activity, in as much as ADA deficiency is associated with a severe combined immunodeficiency disease (29). The present study demonstrates that MTX induces apoptosis of PBL activated by mitogens or superantigens, and may induce clonal deletion of alloreactive T cells at concentrations that are achieved during low-dose clinical treatments (30–32). Remarkably, nonactivated T cells were resistant to apoptosis at up to 1 mM MTX, demonstrating a selectivity toward activated PBL. Furthermore, resting PBL incubated with MTX, and subsequently activated in drug-free medium, underwent apoptosis, suggesting that unactivated T cells may convert MTX to polyglutamate forms that are retained intracellularly. The ability to undergo apoptosis may reflect the capacity of PBL to convert MTX to MTX glu4 and MTX glu5, which were reported to be retained for up to 24 h in breast cancer cells (10, 11). In the present model, maximal apoptosis was achieved after 8 h of exposure to MTX and the half-life of the biological activity of polyglutamated MTX was around 3 d. Such information may be relevant for optimizing dosage and time interval of MTX administration during low-dose treatments. Indeed PBL from MTX-treated rheumatoid arthritis patients underwent apoptosis upon ex vivo activation, although the percentage of apoptotic cells remained lower

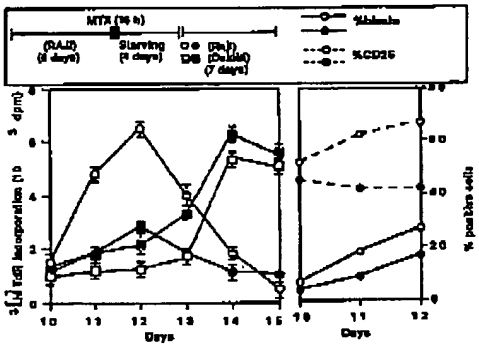

Figure 7. MTX-induced antigen-specific hyporesponsiveness in MLR. Lymphocytes from healthy adult donors were stimulated with RAJI cells with or without MTX (10^{-6} M) added during 16 h at day 6 (*closed or open symbols*, respectively). Lymphocytes were washed and cultured in fresh medium from day 6 to 10, and then restimulated with RAJI or DAKIKI cells. [^{3}H]TdR incorporation in dpm (mean± SD) and CD25 expression and blasts transformation (PACS® analysis) were measured sequentially during 6 d after addition of mitomycin-treated stimulator B cell lines. Results of one representative from three distinct experiments showing similar effects.

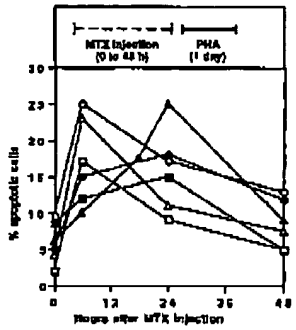

Figure 8. Ex vivo activation of PBL after MTX injection induces apoptosis. Six patients with rheumatoid arthritis under long-term treatment by MTX were studied. Blood samples were taken at indicated times after MTX (7.5–15 mg/kg) intramuscular injection, and PBL were activated by PHA (5 μg/ml). Percentages of apoptotic cells (Hoechst 33342 staining) were determined after 24 h PHA activation. Each symbol represents one patient. Cultures in the presence of folic acid (100 μM) or folinic acid (10 μM) resulted in 1–5% specific apoptosis, as defined in Methods.

than that observed after in vitro incubation of normal PBL with MTX. Nevertheless, uptake and intracellular retention of MTX, along with the irreversibility of its effect on activated lymphocytes, provide a rationale for intermittent administration in contrast with other antiinflammatory and immunosuppressive agents that must be administered daily because of their short half-life and/or reversible activity.

Polyglutamated MTX not only blocks DHFR, the main target enzyme of MTX, but also inhibits a number of folate-dependent enzymes, resulting in inhibition of purine and thymidylate synthesis (10, 11). Complete blockade of DHFR results in the depletion of intracellular reduced folates, but the apoptotic activity of MTX was only reversed at high molar ratios of folic acid to MTX, and much more efficiently by folinic acid, which bypasses DHFR. Complete inhibition by exogenous thymidine supports the prediction that this immunosuppressive activity of MTX may be primarily controlled by intracellular levels of thymidine.

Adenosine accounted for only a minor part of MTX-induced apoptosis because adenosine-triggered apoptosis was restricted to a small subset (~ 15%) of activated PBL. As discussed below, programmed cell death of activated PBL after MTX treatment was initiated at the onset of the S phase of the cell cycle. It may be hypothesized that the apoptotic-triggering signal is generated by altered DNA strands produced by DNA polymerase in the absence of thymidine, thus inducing apoptotic pathways shared by various genotoxic agents, and involving p53, the product of the retinoblastoma susceptibility gene Rb, and multimeric kinase complexes that control the G1/S progression (33–35). However, the apoptotic-signaling pathways induced by altered DNA are still largely unknown. In this respect, it is noteworthy that the antiproliferative properties of MTX demonstrated at high (100 μM) and low (0.01 μM) concentrations are clearly independent from its apoptosis-inducing capacity, suggesting that G1/S progression and apoptosis may be controlled by distinct signaling pathways.

It has recently been reported that several cytotoxic drugs, including MTX, can trigger CD95 (Fas, APO-1) ligand expression in leukemia T cell lines (22) and hepatoma cell lines (36), and that CD95 ligand/CD95 interactions were critical in drug-induced apoptosis. However, in activated PBL, MTX triggered apoptosis by a CD95-independent pathway. This observation is of major relevance for therapeutic applications because CD95-dependent apoptosis is restricted to a subset of preactivated "memory" T cells that express the CD45 RO short isoform (37–39), and that have developed sensitivity to the CD95-mediated apoptosis pathway through an efficient IL-2 signal (23). Conversely, MTX induces apoptosis of naive as well as memory T cells, and may induce deletion of naive activated T cells, as shown by MLR experiments.

MTX is usually used as monotherapy in autoimmune diseases, but in association with other immunosuppressive drugs, such as CsA or FK 506, in graft-versus-host diseases, and organ allograft rejection (4–8). The present study indicates that blocking the IL-2 pathway by inhibition of gene transcription (CsA, FK 506), anti–IL-2 receptor antibodies (CD25) or drugs interfering with IL-2 signaling (rapamycin) that block activated T cells in the G1 phase of the cell cycle, prevents MTX-induced apoptosis in vitro. Therefore, one should not expect synergy between MTX and drugs that interfere with the IL-2 pathway, unless MTX deletes T cells that have escaped activation blockade by the associated drugs. This could be the case in autoimmune disorders where CsA is administrated at much lower doses than in transplantation.

In conclusion, the present study demonstrates that low-dose MTX treatment can induce apoptosis and clonal deletion of activated T cells, and, thus, establishes the potent immunosuppressive properties of this drug. The model provides a rationale for intermittent administration and it could be applied to optimizing the pharmacokinetics of MTX treatment. However, the relative irreversibility of this effect may represent a risk of overimmunosuppression in long-term therapy.

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